Transcriptional activation of mouse cytosolic chaperonin CCT subunit genes by heat shock factors HSF1 and HSF2

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Received 6 September 1999; received in revised form 12 October 1999

Abstract The chaperonin containing TCP-1 (CCT) is a eukaryotic molecular chaperone consisting of eight subunit species and assists in the folding of cytosolic proteins. We show here that all eight mouse CCT subunit genes contain sequences called heat shock elements for binding heat shock transcription factors (HSFs) by electrophoretic mobility shift assays and that these genes are transcriptionally activated by HSFs in reporter gene assays using HeLa cells transiently overexpressing HSFs. These results suggest that HSF1 and/or HSF2 play a role in *Cct* gene expression.

Key words: Chaperonin containing TCP-1; Heat shock factor; Molecular chaperone; Transcription factor; Transcriptional regulation

1. Introduction

The chaperonin containing TCP-1 (CCT, also called TRiC or c-cpn) functions as a heterooligomeric molecular chaperone assisting in protein folding in the cytosol of eukaryotes (reviewed in [1]). It is well known that the CCT isolated from mammalian cells facilitates the folding of actin and tubulin in the presence of ATP in vitro [2–4] and that CCT is bound to newly synthesized actin, tubulin and some other unidentified polypeptides in vivo [5,6]. In budding yeast, CCT is essential for growth, and mutations in individual CCT subunit genes affect the assembly of tubulin and/or actin (reviewed in [7]). CCT is also required for yeast cyclin E maturation [8]. In vitro, CCT assists in the folding of firefly luciferase [9] and transducin [4].

CCT shows a double-torus-like structure with eightfold rotational symmetry constructed from 16 subunits [10]. Eight CCT subunit species of approximately 60 kDa with different p*I*s constitute the CCT complex in mammalian somatic cells. These subunits, α (TCP-1), β, γ, δ, ε, ζ-1, η and θ, are encoded by independent genes: *Ccta* (*Tcp-1*), *Cctb, Cctg, Cctd, Ccte, Cctz-1, Ccth* and *Cctq*, respectively [11,12]. The amino acid sequences of all the CCT subunits contain several highly conserved motifs for ATP binding but the overall identities are only approximately 30%, suggesting that each subunit has both a common ATPase function and a subunit-specific function [1]. Recently, functional cooperativity between different CCT subunits was found in budding yeast and a hierarchy of these subunits in ATP binding activity was proposed [13]. The expression of all the CCT subunit genes is considered to be coregulated to maintain a constant ratio of the eight subunit species [12,14].

In vertebrates, heat shock transcription factors (HSFs) are known to mediate the induction of heat shock proteins (HSPs) by heat and related stress (reviewed in [15]). HSF1 is a major factor mediating the cellular response to heat and related chemical stress [16,17]. HSF1 monomers are distributed in the cytosol under non-stress conditions, and trimers induced by stress are transported to the nucleus to activate the transcription of HSP genes [18,19]. Another HSF called HSF2 has been identified [17,20], and proteasome inhibitors which cause unfolded proteins to accumulate can induce DNA binding activities of HSF2 alone or together with HSF1, depending upon the cell type [21,22]. In addition, HSF2 is considered to play a role in the tissue-specific and developmental stage-specific expression of HSPs [23–26]. Both HSFs bind heat shock elements (HSEs) consisting of nGAAn repeats [27].

Despite the important role that CCT plays in protein folding, little is known about the regulation of *Cct* gene expression. Here we report that all the CCT subunit genes contain HSE sequences for binding HSFs and are transcriptionally activated by HSFs. Both HSF1 and HSF2 bind these sequences in vitro and stimulate *Cct* gene expression in a reporter gene assay with HeLa cells. We discuss possible roles of HSFs in *Cct* gene expression under stress and non-stress conditions.

2. Materials and methods

2.1. Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Human erythroleukemia K562 cells and a transformant cell line of K562 stably expressing HSF2 (clone 86-1 [28]) were cultured in RPMI 1640 medium containing 10% FCS.

2.2. Electrophoretic mobility shift assay (EMSA)

After being washed in PBS and in PBS containing 5 mM NaF and 1 mM Na_3VO_4, cells were lysed in extraction buffer (20 mM HEPES-KOH pH 7.5, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1% NP-40, 500 mM NaCl, 25% glycerol) at 4°C. Following centrifugation (100 000 × *g*, 20 min, 4°C), the whole cell extract in the supernatant fraction was recovered. Double-stranded synthetic oligonucleotides containing HSE motifs (Table 1) were labeled with [^{32}P]dCTP using Klenow fragment, and 4 ng of the labeled probe was mixed with 10 μg of the whole cell extract in 32 μl of reaction buffer (40 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 mM DTT, 8% glycerol, 60 mM NaCl) in the presence of 1 μg of poly(dI-dC). For supershift analysis, 1 μg of

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Abbreviations: CCT, chaperonin containing TCP-1; *Ccta*, *Cctb*, *Cctg*, *Cctd*, *Ccte*, *Cctz-1*, *Ccth* and *Cctq*, genes encoding α, β, γ, δ, ε, ζ-1, η and θ subunits of CCT, respectively; CHBA, constitutive HSE binding activity; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; HSE, heat shock element; HSF, heat shock factor; HSP, heat shock protein; PBS, phosphate-buffered saline; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); TCP-1, *t*-complex polypeptide 1

0014-5793/99/$20.00
PII: S0014-5793(99)01437-4

antibody against HSF1 (Affinity BioReagents, Golden, CO, USA) was included in the reaction mixture. The mixture was incubated for 30 min at room temperature and electrophoresed on 4% polyacrylamide (60:1) gel in TAE at 250 V for 2 h at 4°C.

2.3. Reporter gene assay

Reporter constructs that express full-length firefly luciferase fused with amino-terminal short polypeptides of CCT subunits under control of the mouse *Cct* gene promoters/enhancers (see Fig. 1) were described previously [12]. Effector constructs that constitutively express human HSF1 and HSF2 (pCAHSF1 and pCAHSF2, respectively) were also described previously [28]. HeLa cells (3×10^5) were inoculated in 3.5 cm dishes and cultured in 10% FCS/DMEM for 24 h. These cells were cotransfected with 0.1 μg of the reporter, 1 μg of the effector and 0.1 μg of internal control pRL-SV40 (expressing sea pansy luciferase under the control of SV40 promoter and enhancer; Promega) using 10 μl of LipofectAMINE reagent (Life Technologies). After 5 h exposure to the DNA/LipofectAMINE complex in serum-free DMEM, HeLa cells were further cultured for 19 h in 10% FCS/DMEM. The two different luciferase activities in cell lysates were determined using the Dual Luciferase Assay System (Promega) according to the manufacturer's instructions, and the activity of firefly luciferase was normalized to that of the sea pansy enzyme.

3. Results

3.1. HSE-like sequences found in mouse Cct genes

We previously determined the entire nucleotide sequences of the mouse genomic CCT subunit genes, and analyzed the transcriptional activities of these genes by transient transfection of HeLa cells using reporter gene constructs [12]. Further analysis of the *Ccta* gene transcriptional regulation revealed that the first intron has activity to enhance transcription (data not shown). The first intron was searched for possible enhancer elements using a computer program, and a potential HSF binding sequence consisting of nGAAn repeats, called HSE, was found. We also searched for HSEs in the other mouse *Cct* genes and found HSE-like elements in all eight *Cct* genes (Fig. 1 and Table 1). In the mouse *Cct* genes, the HSE-like sequences are composed of three or four repeats of nGAAn and similar sequences (Table 1). Interestingly, these sequences are found in the 5′ non-coding regions or first introns (Fig. 1). The *Ccta*, *Cctb* and *Cctq* genes contain HSE-like sequences in the first intron, and such sequences in the *Cctg*, *Cctd*, *Cctz-1* and *Ccth* genes were found in the 5′ non-coding region. Although these seven genes contain only one HSE site, there are two such sites in the *Ccte* gene: one in the 5′ non-coding region and the other in the first intron. All nine HSE sequences are distributed within 0.3 kb of the first exon. The regions around the HSE sites are rich in CpG-dinucleotide sequences and often contain potential Sp1 binding sites, suggesting that these regions are transcriptionally active in the cell. The positions of the HSE sites, which are in the first intron or the 5′ non-coding region, are not surprising because human and mouse Hsp90β genes are known to contain HSEs in the first intron and these HSEs show heat shock response [29,30]. The existence of HSE-like sequences in the *Cct* genes suggested that HSFs are involved in the transcriptional control of *Cct* genes.

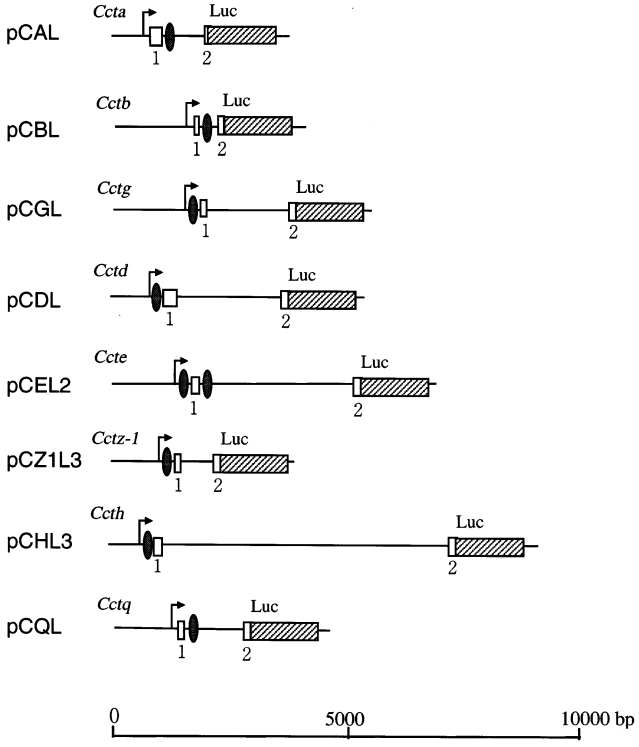

Fig. 1. Localization of HSE elements in reporter constructs expressing firefly luciferase under the control of mouse CCT subunit gene promoters/enhancers. Open boxes indicate introns and the intron numbers are shown below. Shadowed ellipsoid circles indicate HSE-like elements (see Table 1 for their sequences).

3.2. HSF1 and HSF2 bind the HSE sequences found in the Cct genes

To examine whether HSFs can bind the HSE-like sequences found in the *Cct* genes, synthetic oligonucleotide probes containing these elements (Table 1) were produced and analyzed

Table 1
HSE sequences found in the human *HSP70* and mouse *Cct* genes

Gene	Nucleotide sequence of HSE	Probe name
HSP70	GGAGGC**GAA**AC**CCC**TG**GAA**TA**TCC**CC**GAC**CTGGCA	70-HSE
Ccta	GTGTAAGGGAA**GAA**TG**TTC**GA**GAA**TGCACACGGTTG	AHE
Cctb	CCCCAAGTGAA**GAA**GT**TTC**TA**GCA**GGACCTGCTGGC	BHE
Cctg	GCTGCTCTTCT**CTC**CA**GAA**GG**TTC**TGCCGATTCCCC	GHE
Cctd	CCGGCGTCGCT**TTC**TG**GAA**GG**TTC**GT**GGA**GGAGGCG	DHE
Ccte	ACAGCGGCCGA**TTC**CA**GAA**GC**TAC**TCTGCGGCGTAA	EHE1
Ccte	TGTGCTGGCCC**GAA**AG**CTC**GA**GAA**TC**TCC**GGTGCCG	EHE2
Cctz-1	CGGGTGCGTAA**GAC**TT**TTC**GA**GAA**GA**CCC**CGCAGAG	Z1HE
Ccth	ACGTCTCGCCA**GAA**GC**TTC**TG**GGC**AT**TTC**TCCGGCG	HHE
Cctq	ATCGGGATGGA**GAA**GG**TTC**TA**GAA**AGGGAAGCGTGC	QHE

The 70-HSE probe was described by Kroeger et al. [27]. The nucleotide sequences of mouse *Cct* genes were obtained from Kubota et al. [12]. Positions of the HSE sequences in the *Cct* genes are described in Fig. 1, and EHE1 and EHE2 sequences are distributed in the 5′ non-coding region and first intron of *Ccte*, respectively. Sequences highly homologous to the HSE consensus sequence (nGAAn repeat) are shown with underlined bold characters. The sequences described in this table were used in EMSA analysis.

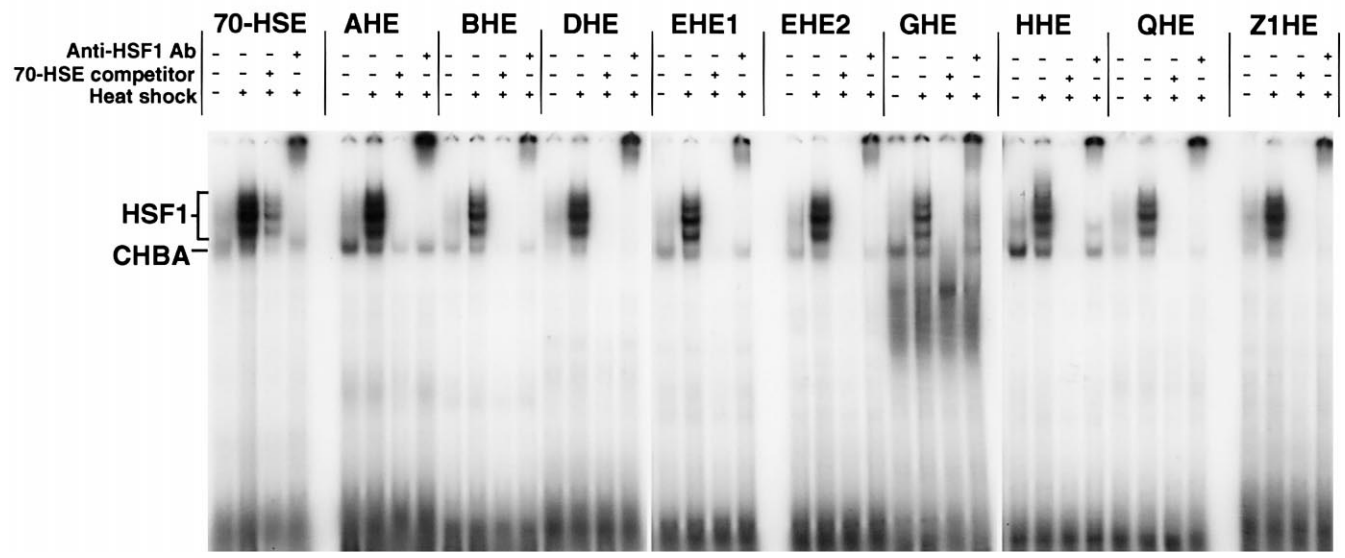

Fig. 2. Binding of human HSF1 to HSE sequences found in the mouse CCT subunit genes. HSE probes of the human *HSP70* and mouse *Cct* genes (see Table 1) were labeled with ^{32}P and incubated with HeLa whole cell extract and analyzed by EMSA. For heat shock, HeLa cells were incubated at 42°C for 1 h prior to extract preparation. For competition and supershift analyses, 100-fold excess non-labeled 70-HSE probe and a mouse monoclonal antibody to human HSF1 were added to the reaction mixture, respectively. CHBA, constitutive HSE binding activity [31,32].

by EMSA. Whole cell extracts were prepared from HeLa cells with or without heat shock treatment, and were mixed with these probes and electrophoresed. As shown in Fig. 2, all probes showed a cluster of several shift bands specific to heat shock treatment and the mobility was the same as that of the human *HSP70* gene HSE probe (designated the 70-HSE probe, see Table 1). The mobility shift pattern showing several bands in a cluster is typical of HSFs [18]. These bands were competed out by 100-fold excess non-labeled 70-HSE probe and were shifted to slowly migrating groups by addition of antibody specific to HSF1 (Fig. 2). It is known that mammalian cells contain a constitutive HSE binding activity (CHBA) under non-stress conditions [31,32], and this activity was also observed in addition to HFS1 in the present study. These results clearly show that HSF1 can bind the HSE sequences of *Cct* genes.

Furthermore, the binding of HSF2 to these HSEs was analyzed by EMSA using whole extracts of wild-type K562 cells and a transformant cell line of K562 stably expressing HSF2 (clone 86-1 [28]). Wild-type K562 expresses little or no HSF2 whereas the transformant expresses a constant level of HSF2 detectable by EMSA and Western blotting [28]. The HSE

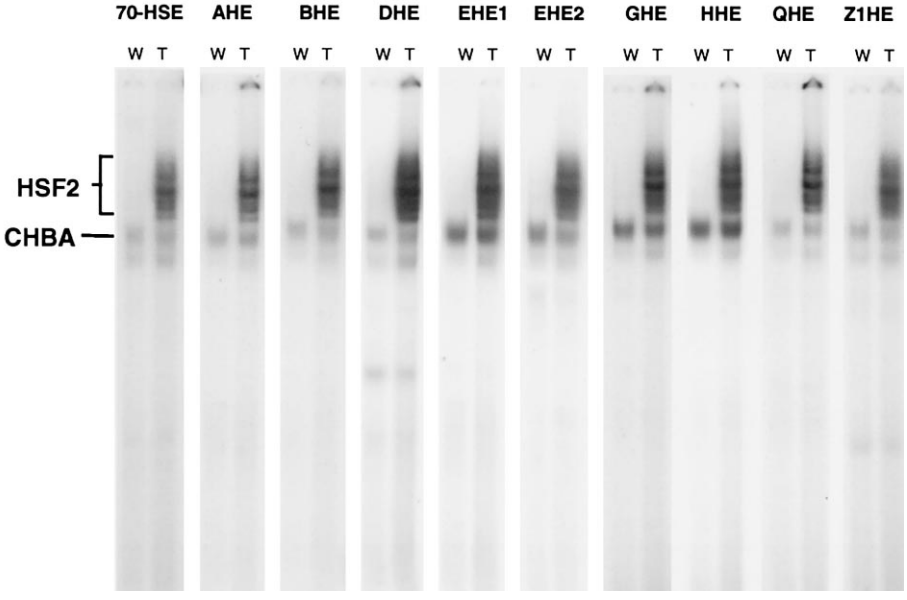

Fig. 3. Binding of human HSF2 to the HSE sequences of the mouse *Cct* genes. The HSE probes of the human *HSP70* and mouse *Cct* genes were incubated with whole cell extracts prepared from wild-type human K562 cells (W) or a transformant cell line of K562 stably expressing human HSF2 (T), and analyzed by EMSA.

Table 2
Activation of the *Cct* gene expression by HSF family transcription factors in the reporter gene assay

Gene	Reporter	Activation by HSF1	Activation by HSF2
Ccta	pCAL	9.59 ± 1.03	8.87 ± 0.32
Cctb	pCBL	9.22 ± 0.16	10.77 ± 0.43
Cctg	pCGL	2.40 ± 0.17	2.15 ± 0.03
Cctd	pCDL	2.08 ± 0.13	1.30 ± 0.05
Ccte	pCEL2	7.90 ± 1.23	7.78 ± 0.39
Cctz-1	pCZ1L3	13.28 ± 2.77	16.12 ± 0.36
Ccth	pCHL3	8.42 ± 0.19	8.24 ± 0.59
Cctq	pCQL	4.80 ± 0.12	6.82 ± 0.24

HeLa cells were cotransfected with three plasmid constructs: the *Cct* firefly luciferase reporter gene construct (see Fig. 1), HSF expression effector plasmid and internal control vector expressing sea pansy luciferase, as described in Section 2. The two luciferase activities of the transfected cells were determined and normalized. The values shown (mean ± S.D. of triplicate experiments) are the fold induction relative to the control effector plasmid with no HSF inserts.

probes of *Cct* genes showed shift bands specific to the transformant by EMSA, and the mobility was the same as that of the 70-HSE probe (Fig. 3). These results indicate that HSF2 is also able to bind the HSEs of *Cct* genes.

3.3. *HSF1 and HSF2 overexpression stimulates Cct gene expression in the reporter gene assay*

To test whether HSF1 and HSF2 can stimulate the expression of *Cct* genes in the cell, reporter gene assays using the reporter constructs expressing firefly luciferase under the control of *Cct* gene promoters/enhancers (Fig. 1) together with the HSF1 or HSF2 expression effector plasmids were carried out. HeLa cells were cotransfected with one of the reporters and each of the effectors. Luciferase activities relative to the vector control with no HSF inserts are shown in Table 2. The transcriptional activities of the reporter genes were 2.1–13.3-fold upregulated by the overexpression of HSF1 and 1.3–16.1-fold upregulated by that of HSF2, depending upon the reporter genes used. The degree of induction for each reporter was similar between the HSF1 and HSF2 effectors. Thus, the potential to activate *Cct* genes seems to be similar between these HSFs. On the other hand, the degree of upregulation substantially varied among the different *Cct* reporters, probably because the transcriptional activities of the reporters themselves in HeLa cells differ widely [12]. Alternatively, the interaction of HSFs with other transcription factors may vary among the different *Cct* genes. Taken together with the results of EMSA analysis, these results indicate that HSF1 and HSF2 are able to activate the *Cct* gene transcription under the set of conditions employed.

4. Discussion

CCT is a member of the chaperonin family of proteins which includes GroEL of bacteria, HSP60 of mitochondria, Rubisco subunit binding protein of plastids and archaea group II chaperonins. The heat shock responses of GroEL [33], HSP60 [34] and archaea group II chaperonins [35] are well characterized, and these proteins act as molecular chaperones to recover proteins denatured by heat shock and related stress including heavy metals and amino acid analogues. The rat *HSP60* gene is known to contain a typical HSE element at the 5′ flanking region [36]. In this report, we demonstrated that all eight *Cct* genes contain HSE elements (Fig. 1 and Table 1) which are recognized by HSF1 and HSF2 in EMSA analysis (Figs. 2 and 3) and that the *Cct* genes are upregulated by these transcription factors in the transient reporter gene assay (Table 1). Since the *HSP60* gene is considered to have evolved from an ancestor common to the CCT subunit genes [1,37], the *Cct* genes appear to maintain a HSE-HSF transcription system similar to *HSP60*. However, the conditions for upregulation may have changed partially during evolution, as described below.

Shena et al. [38] reported that heat treatment at 43°C for 4 h enhanced the expression of *TCP1* (*Ccta*) mRNA in T-cell lymphoma Jurkat cells by 2.4- and 3.8-fold as determined by microarray and Northern blot analyses, respectively. In addition, the level of ciliate CCTγ mRNA was reported to be increased upon $CdCl_2$ treatment [39]. We also observed upregulation of CCT subunit proteins during recovery from chemical stress in mouse and human cultured cells including HeLa cells (submitted for publication). These observations suggest that CCT plays a role during recovery after heat and related chemical stress and that the upregulation of CCT is mediated by the HSE-HSF system. However, no significant increase of CCT subunits in HeLa cells was detected by Western blot analysis during heat treatment at 42–45°C or upon recovery at 37°C, whereas HSP70 protein was induced during the recovery (data not shown). CCT subunits in mouse BALB/3T3 cells also failed to be induced by heat shock treatments (data not shown). CCT was reported not to be upregulated by heat shock in yeasts [40] and *Tetrahymena* [41]. Thus, induction of CCT by stress may be dependent on the type of stress, cells, organisms or other environmental conditions. Since the human *HSP70* gene contains five nGAAn repeats whereas the mouse *Cct* genes contain three to four repeats, the affinities of HSFs to the latter HSEs are possibly lower. The HSEs in *Cct* genes may require more HSF molecules to activate transcription than that in the *HSP70* gene. Alternatively, other transcription factors for *Cct* genes cooperating with HSFs in the cells used in the present study may be inactivated or downregulated by heat shock.

HSF2 is considered to play a role in the tissue-specific and developmental stage-specific expression of HSPs because HSF2 is known to be highly expressed in embryos [23,25] and the testis [24,26]. In mouse embryos, HSF2 is highly expressed at an early stage and the level of expression decreases with development [25]. In the testis, HSF2 is found distributed in the nuclei of pachytene spermatocytes and round spermatids [26]. Similar to HSF2, CCTα/TCP-1 is highly expressed in early stage embryos and less so as development proceeds [42,43]. CCTα/TCP-1 is particularly abundant in pachytene spermatocytes and round spermatids [44,45]. Thus, these observations suggest that HSF2 is involved in the transcriptional control of *Cct* genes in embryos and the testis under non-stress conditions.

Acknowledgements: We thank Drs. A. Nakai and T. Yoshima for helpful discussions.

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